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ORIGINAL ARTICLE/**ARTICOLO ORIGINALE**

Effect of Emdogain coated endodontic materials on viability of human dental pulp stem cells (HDPSCs)

Effetto dei materiali endodontici rivestiti di Emdogain sulla vitalità delle cellule staminali pulpari umane (HDPSCs)

KEYWORDS
Mineral Trioxide Aggregate, Calcium Enriched Mixture Cement, Biodentine, Emdogain, Cell Survival, Human Dental Pulp Stem Cells

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Mineral Trioxide Aggregate, cementi a base di calcio, biodentine, emdogain, vitalità cellulare, cellule pulpari staminali umane

Abstract

Aim: *Biocompatibility is an important characteristic of dental pulp capping agents. This study aimed to assess the effect of mineral trioxide aggregate (MTA), calcium enriched mixture (CEM) cement and Biodentine with/without Emdogain (EMD) on the viability of human dental pulp stem cells (HDPSCs).*
Methodology: *In this in vitro study, HDPSCs were isolated from the root canal of an extracted impacted third molar tooth and cultured. The cells were exposed to freshly prepared endodontic cements in six groups of MTA, MTA plus EMD, CEM cement, CEM cement plus EMD, Biodentine and Biodentine plus EMD in 24-well plates for 24, 48 and 168 hours (6 wells/repetitions for each material group at each time point). Cell viability was evaluated at each time point using the methyl thiazolyl tetrazolium (MTT) assay. Data were analyzed using the Kruskal-Wallis and Mann-Whitney tests.*
Results: *Cell viability was not significantly different at different time points in any endodontic cement group ($p>0.05$) except for CEM cement/EMD group ($p=0.00$). At 24 hours, MTA/EMD and MTA showed the highest cell viability ($p=0.001$). Similar results were obtained at 48 hours ($p=0.000$). At 168 hours, MTA/EMD and CEM cement/EMD showed the highest cell viability ($p=0.000$). Addition of EMD had no significant effect on cell viability in any cement group at 24 or 48 hours. However, addition of EMD to MTA and CEM cement increased the viability of HDPSCs at 168 hours.*
Conclusions: *Addition of EMD to MTA and CEM cement can increase the viability of HDPSCs at 7 days.*

Obiettivo: *la biocompatibilità è una caratteristica importante dei materiali per l'incappucciamento pulpare. Questo studio valutava l'effetto del mineral trioxide aggregate (MTA), del cemento con miscela arricchita di calcio (CEM) e Biodentine con/senza Emdogain (EMD) sulla vitalità delle cellule staminali umane della polpa dentale (HDPSC).*
Metodologia: *in questo studio in vitro le HDPSC sono state isolate dal canale radicolare di un terzo molare estratto e successivamente coltivate. Le cellule sono state esposte, in piastre da 24 pozzetti per 24, 48 e 168 ore, a sei gruppi di cementi endodontici appena preparati: MTA, MTA più EMD, cemento CEM, cemento CEM più EMD, Biodentine e Biodentine più EMD. La vitalità cellulare è stata valutata usando il methyl thiazolyl tetrazolium (MTT) assay. I dati sono stati analizzati utilizzando i test di Kruskal-Wallis e Mann-Whitney.*
Risultati: *la vitalità cellulare non è risultata significativamente differente tra i vari cementi ai tempi analizzati ($p>0,05$), fatta eccezione per il cemento CEM/gruppo EMD ($p=0,00$). A 24 ore, MTA/EMD e MTA hanno mostrato la massima vitalità cellulare ($p=0,001$). Risultati simili sono stati ottenuti a 48 ore ($p=0,000$). A 168 ore, MTA/EMD e CEM cemento/EMD hanno mostrato la massima vitalità cellulare ($p=0,000$). L'aggiunta di EMD non ha avuto effetti significativi sulla vitalità cellulare per nessun gruppo di cemento a 24 o 48 ore. Tuttavia, l'aggiunta di EMD a MTA e cemento CEM ha aumentato la vitalità delle HDPSC a 168 ore.*
Conclusioni: *l'aggiunta di EMD a MTA e CEM può aumentare la vitalità degli HDPSC a 7 giorni.*

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Introduction

Regenerative endodontics refers to tissue engineering procedures to regenerate the lost or damaged tissues such as the dental pulp, dentin or cementum. In this approach, the differentiation potential of human dental pulp stem cells (HDPSCs) or progenitor cells of a mature pulp is employed for regeneration of dental structure (1). Regenerative endodontic procedures were first introduced in 1952 when a type of calcium hydroxide

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cement was used to cover the pulp tissue following vital pulp amputation (2).
Vital pulp therapy minimizes the trauma to the pulp tissue by protecting the pulp against the toxic effects of bacterial products as well as mechanical, thermal and chemical stimuli (3). It seals the pulp tissue and induces the formation of tertiary dentin following reversible pulpitis (3). Vital pulp therapy is performed aiming to cover the pulp tissue with a suitable capping agent and promote the dentinogenesis potential of dental pulp cells (3).
Vital pulp therapy seems to be a superior treatment approach for immature teeth with pulp exposure to allow root development prior to the occurrence of pulp necrosis. In vital pulp therapy, dental materials are placed in direct contact with dental pulp. Regeneration of the dentin-pulp complex following severe trauma involves differentiation of HDPSCs to secondary odontoblasts and subsequent dentinogenesis (3-5). Thus, attempts are ongoing to find an ideal endodontic material for vital pulp therapy.
HDPSCs play a fundamental role in regeneration process by differentiating into odontoblast-like cells. These cells are capable of proliferation and differentiation into different cell lines. The ability of dental materials to enhance differentiation and maturation of these cells determines their biocompatibility (6). In vital pulp therapy, the interaction of HDPSCs with the pulp capping agents affects their proliferation and differentiation capacity (7, 8). An ideal material for pulp capping should be able to trigger the healing process and induce dentin-pulp complex regeneration (9). Several materials have been used as pulp capping agents such as modified glass ionomer, tricalcium phosphate, hydrophilic resins and calcium hydroxide. The success of different pulp capping agents is determined based on the thickness and morphology of the formed dentinal bridge, severity of pulpal inflammation, presence of odontoblasts and biocompatibility of the material (10).
Mineral trioxide aggregate (MTA) is a tricalcium silicate-based cement with more reliable effects compared to those of previously applied materials. MTA is commonly used as a root end filling material and a pulp capping agent and also for perforation repair and apexification (3, 10). MTA induces hard tissue formation in cases with pulp exposure (11). Dentinal bridge formation in presence of MTA occurs faster compared to other materials (11). MTA induces the proliferation of undifferentiated cells for the formation of dentinal bridge by decreasing inflammation (10, 12). Also, direct contact of MTA with HDPSCs causes their differentiation to odontoblast-like cells (13). MTA is commonly used for vital pulp therapy, regenerative endodontic procedures, perforation repair, root end filling and apexification due to its excellent properties such as hydrophilicity, adequate radiopacity, high pH, polymerization expansion, low solubility and optimal biocompatibility (13, 14). However, discoloration potential, difficult handling, presence of toxic compounds in its composition, long working time and high cost are among its drawbacks (15, 16).
Biodentine is a recently introduced tricalcium silicate-based cement with mechanical properties resembling those of dentin (17). It enhances pulp regeneration and mineralization by inducing the formation of reactive dentin and dentinal bridge (18). It is supplied in the form of a powder containing tricalcium silicate, calcium silicate, calcium carbonate and zirconium oxide as opaquer and a liquid containing calcium chloride in an aqueous solvent along with polycarboxylate (19). Its short setting time (a few minutes versus a couple of hours for MTA), superior mechanical properties and optimal sealing ability in contact with dentin are among its favorable properties (20). Biodentine was first introduced as a replacement for dentin and the manufacturer claims that it induces the formation of tertiary dentin. However, it can also be used for vital pulp therapy, perforation repair and as a retrograde root filling material in endodontic surgery (21). It can induce the proliferation and differentiation of HDPSCs and can be used in direct contact with dental pulp in regenerative treatments (22).

Enamel Matrix Derivative (Emdogain/EMD) is a material derived from the pig enamel matrix, Amelogenin is its main constituent. EMD can induce migration, attachment and proliferation of periodontal ligament cells (22, 23) and can efficiently participate in regeneration of cementum, periodontal ligament and bone (24). EMD is believed to exert its effects by provision of extracellular matrix and induction of cell adhesion and differentiation (25). Recently, EMD was proposed for induction of dental pulp regeneration (26, 27). A previous study showed that simultaneous exposure of DPSCs to MTA and EMD results in differentiation of stem cells to odontoblast-like cells, which highlights the synergistic effect of these two materials (28).

Calcium enriched mixture (CEM) cement contains calcium oxide, calcium phosphate, calcium carbonate, calcium silicate, calcium sulfate, calcium hydroxide and calcium chloride, and has clinical properties similar to those of MTA (29, 30). It releases calcium hydroxide during and after setting., it has shorter setting time, higher flow and lower film thickness than MTA, it can form hydroxyapatite by use of intrinsic ions and can be applied in contact with vital pulp in endodontic procedures (29, 31-33).

The toxic effects of endodontic cements on stem cells have been previously studied (28, 34). However, search of the literature yielded no previous study on the comparative effect of CEM cement, MTA and Biodentine with/without EMD on the viability of HDPSCs. Thus, this study aimed to assess and compare the effect of CEM cement, MTA and Biodentine with/without EMD on the viability of HDPSCs.

Materials and Methods

Cell culture

In this in vitro study, each experiment was repeated six times (35, 36). The study was approved in the Ethics Committee of Hamadan University of Medical Sciences IR.UMSHA.REC.1397.61. The HDPSCs were isolated from two surgically extracted impacted caries-free immature third molars of a systemically healthy patient after obtaining informed consent (18-25 years of age). Immediately after extraction, the teeth were rinsed and stored in sterile phosphate buffered saline (PBS) (Gibco, GrandIsland, NY, USA). Stem cells were isolated from the root canals by enzymatic digestion using type I collagenase (2 mg/mL; Worthlington Biomedical, Lakewood, NJ, USA) and were transferred to Dulbecco's modified Eagle's medium (Gibco, GrandIsland, NY, USA). The cells were then cultured again in a culture medium containing 15% fetal bovine serum (Gibco, GrandIsland, NY, USA) and 1% Penicillin/Streptomycin (Gibco, GrandIsland, NY, USA). The culture medium was refreshed every 2-3 days during the process of cell culture. After four passages, a homogenous population of cells was obtained. The surface antigens of HDPSCs were analyzed by flow cytometry (Becton Dickinson, San Jose, CA), with 10,000 events being counted for each case. Cells were trypsinized and incubated in phosphate-buffered saline (PBS) with primary antibodies against CD34 (Biolegend, CA, USA, cat # SC-51540), CD90 (Biolegend, CA, USA, cat #SC-53456), CD105 (Biolegend, CA, USA, cat # SC-71043) as mesenchymal cells marker and CD45 (Biolegend, CA, USA, cat #SC-70686) as hematopoietic marker. Insert plates (pore size: 4.0 µm; SPL Life Science, Gyeonggi-do, South Korea) were used to expose the cells to endodontic cements.

Preparation of endodontic cements

CEM cement (BioniqueDent, Tehran, Iran), Biodentine (Septodont, Saint-Maur-des-Fosses, France) and MTA (Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) were prepared according to the manufacturers' instructions under sterile conditions. Biodentine, MTA and CEM cement were mixed and applied in paraffin wax molds with 10 mm diameter and 1 mm thickness, compressed and incubated at 37 °C and 96% humidity for 10 minutes to set. EMD gel (30 mg/mL and 0.7 mL) (Biora AB, Malmo, Sweden) was diluted with sterile distilled water to obtain 100 µg/mL concentration. The study groups were as follows: (I) Biodentine, (II) Biodentine/EMD, (III) CEM cement, (IV) CEM cement/EMD, (V) MTA, (VI) MTA/EMD, and (VII) control group (cells were not exposed to any material).

Table 1

Comparative effect of Biodentine, Biodentine/EMD, CEM cement, CEM cement/EMD, MTA and MTA/EMD on the viability of HDPSCs at 24, 48 and 168 hours

Groups	24 hours		48 hours		168 hours	
	Mean	Std. deviation	Mean	Std. deviation	Mean	Std. deviation
MTA/EMD	128.68*	31.36	103.25*	7.00	145.64*	19.61
MTA	99.82*	8.23	98.53*	18.99	71.95	30.97
Biodentine/EMD	48.16	11.09	32.10	12.92	27.27	4.13
Biodentine	44.30	21.52	33.31	19.21	40.42	27.52
CEM cement/ EMD	69.85	26.50	39.75	8.75	178.75*	24.44
CEM	38.23	10.78	34.91	11.07	36.15	30.72
P value	0.001		0.000		0.000	

In groups II and IV, the surface of set Biodentine, CEM cement and MTA samples was coated with EMD.

To assess the effect of endodontic cements, about 5,000 cells were cultured in each well of insert 24-well plates (SPL Life Science, Gyeonggi-do, South Korea). The plates allowed indirect contact of materials with cells to prevent cell lysis. After preparation of materials as explained earlier, 1 mg of each material was added to each well of a 24-well plate.

The inserts were removed after 24, 48 and 168 hours (34) and the methyl thiazolyl tetrazolium (MTT) salt (Sigma Aldrich, St. Louis, MO, USA) was added to the wells. The intensity of the produced color in this test correlates with the number of viable cells. The cells were incubated (Binder, NY, USA) for 24 hours. The medium was then replaced and the materials were added to the culture medium. After 24 hours, the plates were removed from the incubator and 10 mL of the MTT solution and 90 mL of alpha-Minimum Essential Medium Eagle supplemented with 10% fetal bovine serum were added to each well. The plates were then incubated at 37 °C for 4 hours. The overlaying medium was gently removed and 100 mL of dimethyl sulfoxide (Gibco BRL, Grand Island, NY, USA) was added to each well. After dissolution of formazan crystals, the optical density was read at 540 to 690 nm wavelength using an ELISA reader (BioTek, VT, USA).

Data were first analyzed using descriptive statistics. The three types of cements were compared in 6 groups using the Kruskal-Wallis test. The Mann Whitney U test was applied for pairwise comparisons. All statistical analyses were carried out using SPSS version 24 (SPSS Inc., IL, USA). P<0.05 was considered statistically significant.

Results

Table 1 shows the effect of different endodontic cements on the viability of DPSCs after 24, 48 and 168 hours of exposure. The Kruskal-Wallis test showed no significant difference in cell viability at different time points in Biodentine (p=0.843), Biodentine/EMG (p=0.98), CEM cement (p=0.979), MTA (p=0.277) or MTA/EMD groups (p=0.132). But the difference in this respect was significant in CEM cement/

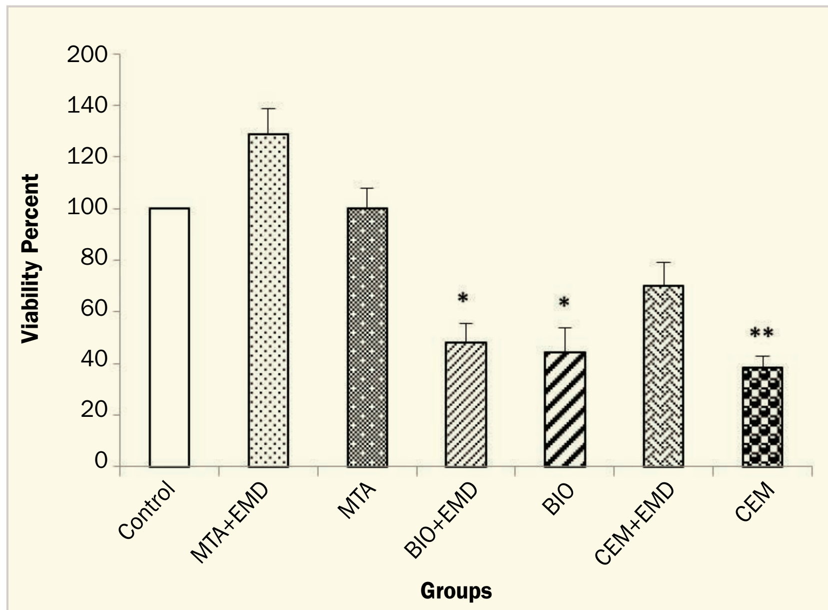
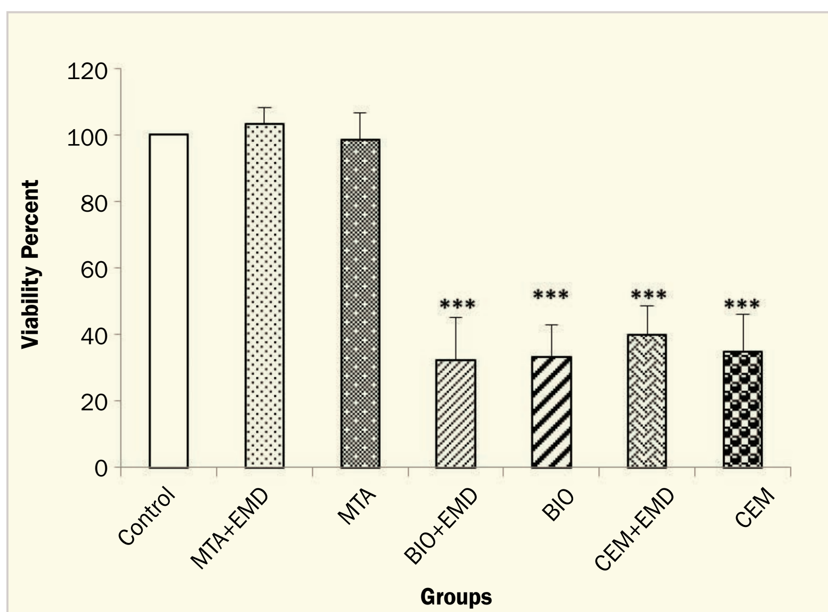


Figure 1
Percentage of HDPSC viability after 24 hours of exposure to endodontic cements

Figure 2
Percentage of HDPSC viability after 48 hours of exposure to endodontic cements



EMD group (p=0.00). Comparison of cell viability following exposure to different materials revealed significant differences among the groups at different time points such that at 24 hours, MTA/EMD and MTA showed the highest cell viability (p=0.001). Similar results were obtained at 48 hours (p=0.000). At 168 hours, MTA/EMD and CEM cement/EMD groups showed the highest cell viability (p=0.000).
Figures 1-3 show the percentage of cell viability in the six groups at 24, 48 and 168 hours. As shown, addition of EMD had no significant effect on cell viability in any cement group at 24 or 48 hours. However, addition of EMD to MTA and CEM cement increased the viability of stem cells at 168 hours.

Discussion

Recently, use of EMD for dental pulp capping has gained attention. Since EMD is in gel form, it does not quickly spread into the pulp tissue (37, 38). Evidence shows that EMD accelerates the formation of reparative dentin and odontoblastic differentiation in pulp capping procedures (39, 40). This study aimed to assess the effect of EMD in combination with commonly used pulp capping agents on the viability of HDPSCs. The results showed that addition of EMD had no significant effect on cell viability in presence of any of the pulp capping agents at 24 or 48 hours. However, after 168 hours (7 days), cell viability in MTA and CEM cement groups significantly increased by the addition of EMD. However, cell viability did not significantly increase in presence of Biodentine/EMD. In general, the highest cell viability was noted in MTA and MTA/EMD groups at 24 and 48 hours while the highest cell viability at 168 hours belonged to the MTA/EMD and CEM cement/EMD groups. The results showed that addition of EMD to MTA and CEM cement resulted in higher level of cell viability; however, this effect became significant after 7 days. A previous study on the toxic effects of endodontic cements namely Biodentine and MTA on mouse fibroblasts revealed that endodontic cements stabilize over time and their toxicity decreases (41). Their findings were in agreement with ours despite the use of a different cell line. Significant effect of addition of EMD to endodontic cements only after 7 days can be due to the fact that cements are toxic during the first 24 and 48 hours but they become chemically stable after 7 days and EMD can then exert its effect.
Our results revealed that at almost all time points, the cell viability in Biodentine and CEM cement groups was less than that in

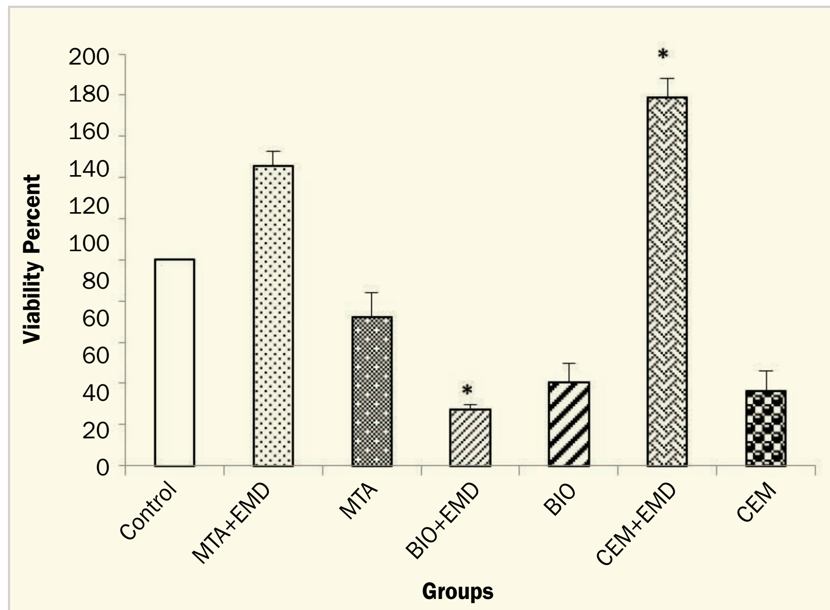


Figure 3
Percentage of HDPSC viability after 168 hours of exposure to endodontic cements

MTA groups. As it has been shown by several investigations, MTA has long term seal, acceptable biocompatibility and dentinal bridge formation which make it the gold standard for vital pulp therapies(42). Similarly, Jung et al. (43) demonstrated that the cell viability in presence of different endodontic cements was not significantly different on day 1 but at 3 days, the cell viability in presence of MTA was significantly greater than that in presence of Biodentine, which was in line with our findings. They measured the amount of heavy metals released from the cements into the surrounding environment and found that the amount of heavy metals released from Biodentine was higher than that released from MTA, which explains the higher cytotoxicity of Biodentine than MTA. They added that Biodentine increased cell viability after 7 days. Another study evaluated the cytotoxicity of Biodentine, glass ionomers and MTA after 1, 3 and 7 days of exposure and concluded that the toxicity of Biodentine and MTA was similar and less than that of glass ionomers (44). This finding was different from our result, which may be attributed to the fact that the cytotoxicity of a material depends on many factors such as the type and concentration of material, passage of time and the type of culture medium used. For example, MSCs derived from dental pulp, periapical cyst, dental follicle and periodontal ligament dreived msc express CD146 as the cell surface antigen. The expression of CD146 level significantly affects on MSCs proliferation and differentiation capacity. Previous study has shown that the number of CD146- positive cells decreases following multiple passage. They found that decreased CD146 expression in human periapical cyst mesenchymal stem cells (hPCy-MSCs) is associated with enhanced cell proliferation, self-renewal, osteogenic differentiation capacity and stemness genes expression (45).

Another factor affects the survival and differentiation of stem cells is the type of scaffold in tissue engineering which was not a variable in this study (46).

In our study, the cytotoxicity of MTA was lower than that of Biodentine and CEM cement at all time points. Saberi et al. (34) evaluated the toxic effects of Biodentine, CEM cement, octacalcium phosphate and MTA on stem cells of the apical papilla. They found that the cytotoxicity of CEM cement was the highest after 24 hours while MTA showed the highest cytotoxicity at 48 and 168 hours. It should be noted that at 168 hours, CEM cement showed the lowest cytotoxicity. In our study, CEM cement/EMD and MTA/EMD showed the least cytotoxicity at 168 hours. They explained that application of calcium silicate-based cements leads to continuous formation of calcium silicate hydrate and deposition of calcium carbonate phosphate. Release of calcium ions can cause toxic inflammatory reactions. On the other hand, release of this ion from silicate cements is important for the viability of mesenchymal stem cells (19). This ion is capable of signaling and plays an important role in regulation of cellular activities. The migration of stem cells is also affected by calcium ions (47, 48). It has been shown that Calcium silicate cements continuously release calcium ions (49). CEM cement, MTA and Biodentine are among the calcium silicate-based cements. Thus, difference in cell viability in presence of different cements at different time points may be due to the release of calcium ions, which has a double action. In our

study, the highest cell viability was noted in MTA and MTA/EMD groups at 24 and 48 hours and CEM cement/EMD group at 168 hours, which indicates that at almost all time points, groups with EMD had the least cytotoxicity and highest cell viability. However, this effect was only significant at 7 days. As explained earlier, it can be due to the high cytotoxicity of materials at 24 and 48 hours, which would mask the efficacy of EMD. On the other hand positive effect of EMD in combination with endodontic cements is dependent on its molecular mechanisms and effects on growth factors releasing (50).

The current results highlight the efficacy of EMD in combination with MTA and CEM cement to increase the viability of HDPSCs. Future studies are required to assess the effect of addition of EMD to endodontic cements on the viability of other cell lines. This study had an in vitro design. Thus, generalization of results to the clinical setting must be done with caution. Further clinical studies are required to assess the efficacy of these compounds in the clinical setting.

Conclusions

Within the limitations of this in vitro study, the results showed lower cell viability in presence of Biodentine compared to MTA and CEM cement. Addition of EMD to endodontic cements had no significant effect on the viability of DPSCs at 24 or 48 hours but it significantly increased cell viability in presence of MTA and CEM cement at 7 days. For further experiments more dental pulp to obtain more MSCs is needed. Data from the present study showed that EMD can be used with endodontic cements in vital pulp therapies and regenerative endodontic treatments, but studies evaluating the in vivo effect of EMD coated cements on HDPSCs viability are needed.

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Conflict of interest

The authors deny any conflict of interest.

Clinical relevance

Stem cells play an important role in regenerative endodontic, so it is critical to enhance viability and differentiation of these cells. Coating endodontic materials with Emdogain increased cell viability and can be used in vital pulp therapies and regenerative endodontic treatments.

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